Metagenomics: Microbial diversity through a scratched lens

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Abstract

Since nucleic acids were first extracted directly from the environment and sequenced, metagenomics has grown to one of the most data-rich and pervasive techniques for understanding the taxonomic and functional diversity of microbial communities. In the last decade, cheaper sequencing has democratized the application of metagenomics and generated billions of reads, revealing staggering microbial diversity and functional complexity. However, cheaper sequencing has come at the cost of reduced sequence length, resulting in poor gene annotation and overestimates of bacterial richness and abundance. Recent improvements in sequencing technology are beginning to provide reads of sufficient length for accurate annotation and assembly of whole operons and beyond, that will once again enable experimental testing of gene function and re-capture the early successes of metagenomic investigations.

Diversity in sharp focus

The revelation of the ‘Great Plate-Count Anomaly’ by Staley and Konopka in 1985 [1] highlighted that contemporary understanding of microbial metabolism was highly skewed towards a small fraction of readily culturable bacteria. To address this issue, direct extraction and cloning of environmental DNA began to unravel novel phylogenetic [2,3] and functional [4] diversity. In 1998, the term ‘metagenomics’ was coined [5] and this exciting new field hinted at the scale of

genetic variability within natural microbial populations [6] and associated phage [7]. In 2000, Béjà et al. [8] cloned 130-kb environmental DNA fragments from seawater into BAC libraries and found bacteriorhodopsin, a mechanism for ATP generation from light. This type of photochemistry previously had been know to occur only in hypersaline ponds; its discovery in the oceans is perhaps one of the most heralded successes of metagenomics [9]. Four years later, two landmark studies demonstrated the power of metagenomics to explore environmental microbiology: Venter et al. exposed the magnitude of microbial diversity in the surface water of Sargasso Sea, identifying 148 novel phylotypes and 1.2 million novel genes in a single study [10]; Tyson et al. demonstrated that when diversity was low, metagenomics could be used to reconstruct genomes of uncultured bacteria to reveal complete metabolic pathways, providing insight into their nutritional requirements and biogeochemical functions [11]. Encouraged by the success of their previous exploration of marine microbial diversity, Venter expanded their previous study on a global scale. In 2007, the first results of the Global Ocean Survey (GOS) were published [12], revealing ~390 new species and ~6 million predicted protein sequences in ~4000 protein clusters of which 42.6% had no known homology [13]. The unprecedented volumes of data generated by this project catalyzed the advancement of the bioinformatic techniques required to assemble, analyze and contextualize novel genes, phyla and pathways. Subsequent and ongoing voyages ensure that the GOS project remains to this day the largest metagenomic survey undertaken.

An era of open-access science

Early adopters of metagenomics had the foresight to understand that the datasets from a single metagenomic investigation were too large for comprehensive analysis by a single research group and opened up datasets for public access, often prior to initial publication. This in turn fuelled the development of third-party tools for data management and analysis (e.g. MG-RAST [14], CAMERA [15], IMG-ER [16]) as well as standards for collection of metadata to assist in downstream analysis [17]. At the current time (February 2012), MG-RAST currently holds 111 publically available metagenomic projects,

comprising 7,444 datasets, 459 million sequences and 8.4×10^{10} base pairs. Public availability of metagenomic datasets has enabled a broad range of bioinformatic investigations into novel clades, genetic diversity and microbial pan-genomes [18-22]. Fragment recruitment of metagenomic fragments to full genome sequences from related isolates has highlighted the prevalence of 'hypervariable regions' (HVRs) across multiple strains and species, where the genomic content of the sequenced isolate is not representative of the population as a whole [12,20,23] (Grote et al., *in submission*). Comparative metagenomic studies have shed light on community adaptation to a local environment, particularly in nutrient cycling (e.g. [24-28]), with new algorithms to deal with the statistical challenges of large numbers of observations with minimal or no replication [29-31] (Beszteri et al., *in submission.)*. Whereas the first culture-independent investigations into microbial diversity were performed on 38 16S rRNA clones [2], amplification of DNA with barcoded primers [32] to allow sequencing of multiple samples in a single run, coupled with massively reduced sequencing costs enabled investigators to identify tens of thousands of unique sequences [33] in a single study, providing an unprecedented insight into microbial diversity. Large-scale projects such as the Earth Microbiome Project (http://www.earthmicrobiome.org/) and *Tara* Oceans Expedition [34] continue to explore environmental microbial diversity with rich metadata to better model and understand microbial ecology at the systems level. Similar programs to map the human microbiome are also ongoing [35,36], making metagenomics one of the most data-rich, fastest-growing and exhaustively reviewed scientific fields [9].

Data rich, information-poor: The cost of metagenomic democratization

Until 2006, metagenomics had required the cloning of environmental DNA into vectors and their subsequent Sanger sequencing and fragment assembly. With costs of Sanger sequencing approaching ~$500 per Mb [37], large scale metagenomics projects were limited to those with significant financial resources. Use of replicated experimental samples was extremely limited, preventing statistically rigorous analysis of biological variation and correlation of taxonomic

abundance with nutrient metadata [38]. The pairing of metagenomics with an emergent pyrosequencing technology [39] marked the beginning of a sequencing revolution. In rapid succession 454 (Roche), Illumina, SOLiD (Life Technologies) and Pacific Biosciences drove the cost of sequencing down to < $0.10 per Mb (Fig. 1) (albeit with no similar decrease in the cost of data storage and computation [40]), democratizing metagenomic analysis, with a concomitant explosion of studies across a wide range of environments to investigate the diversity and functional capacity of all domains of life (e.g. *Eukarya*: [41,42]; *Archaea*: [4,43]; *Bacteria* [21,33,36,44,45]) and their associated viral communities [46-49], even from as bizarre a locale as the windshield splatter from a single road trip [50]. However, second generation sequencers generated reads ranging from ~35-500 bp depending on the technology used – far shorter than the 130 kb fragment used to identify the 747 bp gene encoding proteorhodopsin by Béjà et al., or even the length of Sanger sequences used in the GOS study (~1000 bp). Furthermore, each advance in sequencing technology introduced its own biases, which, coupled with non-standardized metadata collection, made it difficult to compare the results of one investigation to previous datasets. Despite the reduction in sequencing costs, use of replicated samples was still poor [38]. In 16S rRNA diversity studies, the reduced sequence length of pyrosequencing prevented the use of full-length sequences; relying instead on shorter hypervariable regions (V1 through V8) sequenced both separately and in combination. Species richness of samples was significantly biased by primer choice, with V6 and V1+V2 overestimating richness while V3, V7 and V7+V8 underestimated richness [51]. Issues of over-estimation of the 'rare' biosphere from sequencing error [52-54] and PCR chimeras [55] required correction by post-sequencing analysis with tools such as AmpliconNoise [56]. Bias introduced during sample preparation, such as variation in DNA extraction efficiencies of different taxa [57] and poor representation of low G+C taxa [58,59] required fine-tuning of experimental design and greater use of controls.

In shotgun metagenomic studies, annotation of genes is improved by inclusion of identifying features such as promotors, riboswitches, co-operonic genes and signature protein domains. The probability of capturing such features on the same fragment is proportional to the length of the fragment. Separation of genes from their distinguishing features is significant in Sanger sequences (~1000 bp) compared to BAC sequences (~130 kbp). With the even shorter lengths of pyrosequencing, the issue is exacerbated. Furthermore, increased sequencing errors of ~1-3% (depending on sequencing technology) frequently introduced frameshift mutations into reads [60]. Consequently, the number of identifiable homologs on shorter reads was 20-30% lower than for Sanger reads from a bacterial metagenome and 70% lower for viral metagenomic samples [61]. As early as 2008, Wommack et al. concluded that despite the reduced cost per basepair of pyrosequencing, the cost per unit of information was comparable with Sanger sequencing [61]. Whilst the continued reduction of sequencing costs has tilted the cost-benefit balance firmly in favor of next-generation sequencing, the issues of annotating short fragments remain [48,62]. It is no coincidence that the ubiquitous contextualization of the ecological significance of novel, experimentally derived microbial function overwhelmingly choose the GOS and longer read datasets for their analyses [63-66]. Furthermore, the rate of discovery of putative protein sequence has dwarfed the rate at which protein structure and function can be characterized, and has made manual curation of functional genes unfeasible (Fig. 1). Of the 20.6 million protein sequences in the current (2012_03) release of the UniProt database, only 2.8% have had their existence confirmed either at the protein or the transcript level (http://www.ebi.ac.uk/uniprot/TrEMBLstats/). Instead, automated annotation via homology transfer from similar sequences with known function was favored. However, homology and sequence similarity are not synonymous. As the number of automated annotations of increasingly diverse putative proteins continues to increase exponentially, transfer of homology has resulted in ‘homology creep’ to non-homologous sequences [67]. Automated annotation can be drastically improved via incorporation of a measure of evolutionary distance

(phylogenomics) but requires near-full length protein sequences [68]. Only very recently has sequencing technology improved sufficiently to approach such lengths (~700-1100 bp) [69], whilst maintaining sufficient coverage to allow shotgun metagenomics to achieve its full potential.

Despite dramatic increases in sequencing efficiency (Fig. 1), inadequate coverage, which translates into “under sampling”, remains a major issue in metagenomics. Under sampling compromises some of the favorite experimental design strategies of ecologists, who often seek to understand how functional aspects of microbial communities vary along clines, such as gradients in latitude, temperature, or productivity. Under sampling can be ameliorated by binning data at courser resolution, for example, by COG instead of by species. However, such a shift in strategy elicits a significant cost - the loss of genome context, which is often crucial for interpreting function.

Recapturing lost information: assembly and SAGs

To overcome the effects of short reads on accurate annotation, assembly of fragments into longer contigs has been attempted. Whilst numerous assembly programs have successfully reconstructed genomes from clonal organisms [70], successful assembly of contigs from metagenomic data is more limited. Problems with metagenomic assembly arise from poor community coverage [71], significant genomic variance in natural populations [12,20] and a high risk of chimeric sequence generation [62,72,73]. Furthermore, repetitive reads present a dichotomy for metagenome assembly [74]. During the assembly of a clonal genome, repetitive reads are problematic during construction of de Brujin graphs and are therefore removed from analysis [75,76]. Conversely, in a metagenome, repetitive reads are likely to come from higher coverage of dominant organisms and should therefore be assembled together. To tackle this issue, new ‘digital normalization’ algorithms to remove redundant reads and improve assembly are now emerging [77]. The likelihood of meaningful assembly is directly related to the complexity of the sampled community and the genetic variability of its constituents. In relatively simple systems, a combination of deep sequencing with

short reads combined with longer reads for fragment recruitment and robust chimera checking are yielding some successes in assembling both microbial [11,62,78-80] and viral [81] genomes. Even with long reads, assemblies of metagenomes from complex communities (>400 taxa) are problematic. However, the longer reads have an inherent advantage of capturing full gene information for more accurate annotation [62]. The emergence of new metagenome assembly tools such as MetaORFA [82], Genovo [83], MetaVelvet [84], Meta-IDBA [85] and SEAStAR [86] may improve the utility of short sequences in metagenomes from complex communities.

To circumvent assembly problems most metagenomic studies rely heavily on complete or draft genomes to identify fragmentary sequences and interpret natural variation in an organismal context. Many online tools, e.g. MG-RAST, use this strategy, and have limited power to resolve metagenomic data originating from uncharted sectors of microbial diversity. Advances in culturing technology have made more genomes from relevant organisms available, but the uncultured part of microbial diversity remains substantial. To bridge this gap, researchers are increasingly turning to single cell genome amplification. Individual cells are isolated either by micromanipulation or by fluorescent-activated cell sorting (FACS) flow cytometry before lysis and multiple displacement amplification (MDA) of DNA to concentrations suitable for sequencing [87]. DNA can then be deeply sequenced with short-read, high throughput sequencing and readily assembled into large contigs, often assisted by scaffolding using longer Sanger reads [88,89]. Single-cell genomics is a significant advance that is providing draft genomes from organisms, many of them important, that have so far evaded cultivation. This technique was recently used to sequence the genome of SAR86, an important, highly abundant, but as-yet uncultured marine aerobic chemoheterotroph [90]. With ever-decreasing sequencing costs and rapid FACS cytometry cell isolation, it is not difficult to imagine that high-throughput metagenomics of important community representatives, and/or populations within a community from single-cell amplified genomes is imminent. Such an approach

will avoid issues of chimeric assemblies (other than contaminants) and will enable functional annotation with intact synteny. Amplification-free, single-molecule DNA sequencing technologies such as those implemented in the MinION™ and GridION™ (Oxford Nanopore) and PacBio *RS* (Pacific Biosciences) will further reduce the cost, simplify assembly and improve the accuracy of single-cell metagenomics, perhaps even removing the need for cell isolation entirely. Accurate assembly of genomes from single cells and/or reads long enough to contain complete operons will have two major advantages. Firstly, complete genes will enable fine-scale intra and inter-specific phylogenomic analyses, improving our understanding of community structure and how bacterial diversity is derived and maintained via periodic selection [91] and viral predation [23,92,93]. Secondly, accurate annotation of genes will dramatically improve the capacity of systems biologists to model community connectivity and thus the effects of perturbations on microbial biogeochemical processes [94].

The continued importance of culturing

Accurate annotation may provide insight into the metabolic potential of an organism and its community. However, it is often difficult to predict the ecological significance of an annotated gene without first considering its function in the broad context of metabolism. In some cases, this can be achieved by reconstructing metabolic pathways and testing predictions by experimentation with axenic cultures. The demonstration that SAR11 bacteria are methylovores was an example of this strategy [64]. In other cases, particularly those in which a gene is functioning in non-canonical pathways that are not represented in KEGG of other databases, the only choice may be exploratory experiments with cells in culture. SAR11 proteorhodopsin provides an apt example. Whilst the abundance and biochemistry of proteorhodopsin as an ATP-generating proton pump had previously been described from metagenomic data, exposure to light did not significantly improve the growth of SAR11 in axenic cultures, as would have been predicted from the annotated function. A decade after proteorhodopsin was first reported in metagenomic datasets, Steindler et al. showed that it provides an important source of ATP under conditions of carbon starvation, with cells grown

in the dark forced to consume endogenous reserves of carbon for survival [95]. None of this surprises the average genome scientist, who by now, accustomed to the principles of systems biology, understands that selection is acting to shape the output from genes functioning in a concerted way.

The 'post-*Beagle*' era of metagenomics

There is little doubt that metagenomics has revolutionized our perspective of microbial taxonomic and functional diversity, and the scale of the generation of testable hypotheses from patterns observed through metagenomic studies has led to favorable comparisons with Darwin's *Beagle* voyage [96]. However, like *The Origin of Species*, the power of observational science lies not in the data collection, but in the analysis and experimentation. After his four-year voyage on the *Beagle,* Darwin's *magnum opus* resulted from two decades of experimental evidence to test his hypotheses on common ancestry, convergent evolution and descent with modification [97]. Similarly, the discovery of proteorhodopsin by Béjà et al. in marine metagenomic datasets was more potent for its confirmation through *in vitro* cloning and purification from complete gene sequences on 130-kb fragments [8]. Although early metagenomic investigations with pyrosequencing provided more data, the increased error rates in sequencing, assembly and annotation would have made the success story of bacteriorhodopsin less likely. Darwin's lack of training as an ornithologist resulted in erroneous classification of some Galápagos finches as blackbirds and required careful curation by John Gould before the true extent of adaptive selection was apparent [97,98]. Analogously, genomic fragments annotated via transfer of homology from similar sequences with known function suffer from a similar issue. Greater efforts in novel protein curation are required for accurate predictions of taxonomic and functional diversity to better elucidate their roles in biogeochemical cycling. It is worth remembering, however, that metagenomics is still in its infancy and that the difficulties of short read lengths and fragmented genes are likely to be transient. Improvements to sequencing biochemistry and new methodologies are now increasing read lengths to a critical point where

near-complete genes can be captured on a single read whilst maintaining depth of coverage, significantly improving annotation even when assembly into full genomes is difficult. Lessons have been learned for replicated experimental design to allow for robust statistical analysis and standards for metadata collection will improve comparisons between datasets [38,99]. Broad, shallow, replicated sequencing across large numbers of samples, followed by targeted deep sequencing and single-cell genomics will allow investigators to define a hypothesis; identify samples most likely to provide insight and then identify metabolic potential within single genomes and the community as a whole [38]. Confirmation of predicted biochemistry in axenic and community cultures of important taxa [100] will improve our ability to more accurately predict, contextualize and, importantly, test novel function and its role within bacterial populations and their wider communities, and will continue to drive the metagenomic revolution in microbial ecology.

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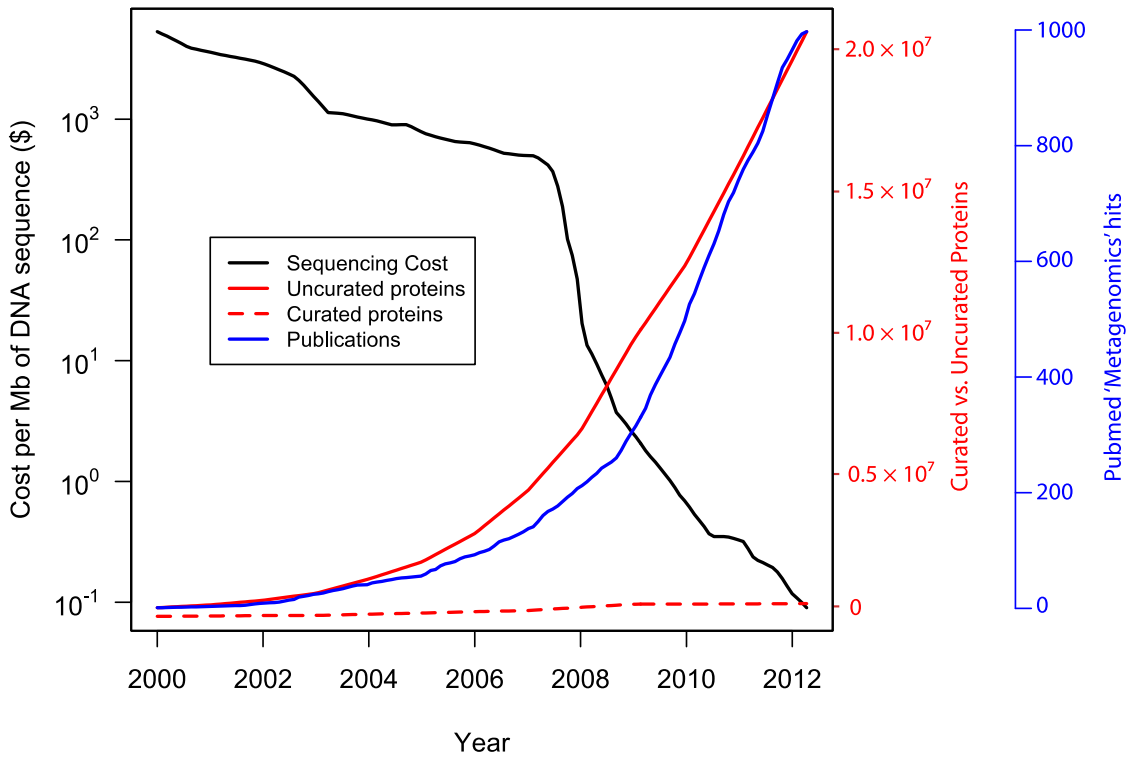

Figure 1 - Cost of DNA sequencing and its impact on genomics and metagenomics. Y-axis 1 (black): The cost per Mb of DNA sequencing on a log scale (data from http://www.genome.gov/sequencingcosts/). Y-axis 2 (red): The total number of sequences in the UniProt (http://www.uniprot.org/) database for automatically annotated (solid, TrEMBL database) and manually annotated (dashed, SwissProt database) proteins (data courtesy of Predrag Radivojac). Y-axis 3 (blue): The total number of metagenomics publications in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). The search term "metagenomics"[MeSH Terms] OR "metagenomics" was used to retrieve publication records in XML-format and binned by month and year according to the 'DateCreated' element.